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(54) Title: DETECTION OF ANALYTES USING FLUORESCENT ENERGY TRANSFER

(57) Abstract

The invention provides labeled proteins suitable for determining the presence of cAMP, other second messengers and organic molecules. The proteins are separately labeled with fluorochromes which, when in close spatial proximity, preferably, less than about 6 nm, interact through the transfer of energy from one fluorochrome to the other. A composition of matter $(S_1 kA)_{n1}(S_2 kD)_{n2}$ is provided wherein S_1 and S_2 are two proteins which are associated in one state and substantially disassociated in another, the equilibrium between which is controlled by the free concentration of an analyte, and A and D are fluorochromes, the emission wavelength of fluorochrome D overlapping the excitation wavelength of fluorochrome A and the distance between A and D being in sufficiently close proximity to allow radiationless transfer of energy between the fluorochromes. A and D can be selected from a variety of acceptor, donor pairs such as fluorescein and tetramethylrhodamine.

DETECTION OF ANALYTES
USING FLUORESCENT ENERGY TRANSFER

                    BACKGROUND OF THE INVENTION

10   This invention relates to energy transfer between
     fluorochrome labeled proteins to determine analyte
     concentration and, more particularly, to methods of
     determining cAMP concentration using labeled cAMP dependent
     protein kinase.

15   The function of many biological molecules is mediated
     through interactions with proteins and protein complexes.
     Many of these molecules are found in extremely small
     concentrations within a living cell.   However, their
     concentration may transiently change in response to
20   physiological stimuli. In many instances, it is important
     to determine the concentration of such molecules to assess
     the metabolic state of a cell, hormonal responses and
     certain diseases. Even if the level changes several fold,
     at such minute concentrations, such change is not
25   detectable.

        One such important molecule which mediates biological
     function through protein interaction is cyclic AMP (cAMP,
     adenosine 3', 5' - cyclic monophosphate).   Cyclic AMP
     regulates intracellular reactions in all procaryotic and
30   nucleated animal cells that have been studied to date. It
     functions as a ubiquitous intracellular mediator, or second

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messenger, of a variety of hormone induced effects, the
first messenger being the extracellular hormone.   Such
hormone induced effects mediated by increased cAMP levels
include, for example, triglyceride breakdown in a fat
5 tissue induced by epinephrine, adrenocorticotropic hormone
(ACTH), glucagon or thyroid-stimulating hormone (TSH);
water resorption in the kidney by vasopressin; glycogen
breakdown in the muscle and liver; increased heart rate by
epinephrine and progesterone secretion by the ovary in
10 response to luteinizing hormone.   These differential
responses to cAMP are induced by hormone-receptor
interactions at the cell surface and result in the
intracellular synthesis of cAMP.   Thus, different target
cells respond to increases in cAMP levels in different but
15 characteristic way.

For cAMP to function as a second messenger, its
intracellular concentration must be tightly controlled and
able to change rapidly in response to hormone-receptor
binding.   Normally, cAMP levels are less than about 1 μM
20 and increase approximately five-fold upon hormonal
stimulation.   The increase in cAMP levels is due to
synthesis by the enzyme adenylate cyclase from ATP.
Following this transient increase, cAMP levels are rapidly
returned to normal levels through the action of
25 phosphodiesterases.

To effectively study cAMP dependent hormonal responses
and their physiological mechanisms, it is necessary to
accurately measure small concentrations of cAMP.   More
essentially, it is important to measure free cAMP
30 concentrations, since in general, the biological activity
of intracellular messengers is controlled by and correlates
with the concentrations of free messenger, whereas total
concentrations measured after tissue disruption include
material bound to biologically irrelevant sites.   Previous
35 methods   for   measuring   cAMP   concentrations   employed

classical   competitive   binding   assays   such   as
radioimmunoassays,   and   required   the   manipulation   and
preparation   of   extracts   from   thousands   or   millions   of
cells.   These methods resulted in poor spatial and temporal
5   resolution   and   were   incapable of   distinguishing   free   or
biologically active cAMP levels from total or sequestered
cAMP.   Moreover,   these   methods   required   the   use   of
hazardous,   perishable   isotopes   and   were   therefore
expensive.

10   Additionally, second messengers other than cAMP such
as cyclic GMP (cGMP), calcium and diacylglycerol as well as
other organic molecules such as tumor-promoting phorbol
esters are of great importance in signal transduction and
cell   physiology.   Accurate   determination   of   the
15   biologically active concentrations of these molecules is
also necessary to effectively study hormonal responses and
physiological   mechanisms.   However,   except   for   simple
inorganic ions such as calcium, there is no indicator
system   such   as   to   measure   the   biologically   active
20   concentrations   of   these   molecules.   Most   of   the   same
drawbacks   that   exist   for   determining   free   cAMP
concentrations within the cell exist for these molecules as
well.

        Thus,   there   exists   a   need   for   a   method   to   rapidly,
25   efficiently   and   non-destructively   measure   free
intracellular cAMP concentrations as well as other organic
molecules in single living cells.   Such an invention would
be of critical importance to the understanding of hormonal
regulation.   The present invention satisfies this need and
30   provides related advantages as well.

                        SUMMARY OF THE INVENTION

        The invention provides labeled proteins suitable for
determining the presence of cAMP, other second messengers

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and organic molecules. The proteins are separately labeled with fluorochromes which, when in close spatial proximity, preferably, less than about 6 nm, interact through the transfer of energy from one fluorochrome to the other.

5   A composition of matter, $(S_1 \kappa A)_{n1} (S_2 \kappa D)_{n2}$ is provided wherein S_1 and S_2 are two proteins which are associated in one state and substantially disassociated in another, the equilibrium between which is controlled by the free concentration of an analyte, and A and D are fluorochromes, 10 the emission wavelength of fluorochrome D overlapping the excitation wavelength of fluorochrome A and the distance between A and D being in sufficiently close proximity to allow the radiationless transfer of energy between the fluorochromes. A and D can be selected from a variety of 15 acceptor,   donor   pairs   such   as   fluorescein   and tetramethylrhodamine   and   derivatives   thereof.   The concentration of analytes such as cAMP in a sample can be determined by contacting the sample with $(S_1 \kappa A)_{n1} (S_2 \kappa D)_{n2}$ providing energy near the excitation wavelength of D and 20 measuring the fluorescence of A or D, the concentration of cAMP and other said analytes being determined by the ratio of emission of D to the emission of A, previously calibrated with reference solutions of known analyte concentration.

25   BRIEF DESCRIPTION OF THE DRAWINGS

        Figure 1 is a schematic diagram depicting the use of fluorescence energy transfer to measure cAMP.

        Figure 2 shows the emission spectrum of labeled cAMPdPK at various concentrations of cAMP and the inset 30 plots the ratio of emission at 520 nm to 580 nm at the various cAMP concentrations.

Figure 3 shows the increase in cAMP concentration when a smooth muscle cell is treated with a membrane permeable cAMP analog.

Figure 4 shows the measurement of cAMP within a single
5 cell when treated with various β_2-adrenergic agonists and antagonists.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "protein" or "subunit" indicates a linear sequence of amino acids which exhibits
10 biological function. This linear sequence includes native proteins, domains of proteins and fragments of proteins so long as function is maintained. The term also includes polypeptides and peptides.

As used herein, the term "associated" refers to two
15 proteins such as subunits of an enzyme, which are in contact with each other. The contact region may include all or parts of the two molecules. Therefore, the term "substantially disassociated" or "disassociated" refers to the loss of contact between the associated regions,
20 including loss of all contact regions such that the proteins are completely separated as well as the loss of some regions so that the body of the proteins are no longer in close proximity to each other but may still be tethered together. The term "complex" as used herein, refers to the
25 proteins or subunits when they are in the associated state. The complex may include more than one molecule of each of the two proteins.

As used herein, the term "cAMPdPK" refers to the cyclic AMP dependent protein complex as described in
30 Alberts et al., (1989), Molecular Biology of the Cell, Garland Publishing, Inc., and in Slice and Taylor, (1989),

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J. Biol. Chem. 264:20940-20946, both of which are herein
incorporated by reference. The complex consists of two
proteins which perform regulatory and catalytic functions.
It is intended that the term will include all isotypes of
5   the regulatory and catalytic proteins.

As used herein, the term "fluorochrome" refers to a
molecule which is capable of absorbing energy at a certain
wavelength range and releasing the energy at a wavelength
range other than the absorbance range. Therefore, the term
10 "excitation wavelength" refers to the range of wavelengths
that a fluorochrome absorbs energy. The term "emission
wavelength" includes to the range of wavelengths that a
fluorochrome releases energy or fluoresces. The term
fluorochrome also includes derivatives thereof, such as
15 chemical moieties for attachment to proteins. Examples of
fluorochromes are fluorescein and tetramethylrhodamine. A
derivative of each is fluorescein isothiocyanate and
tetramethylrhodamine isothiocyanate.

As used herein, the term "sample" refers to a
20 material, such as a solution, extract or intact cell
suspected of containing an analyte.

As used herein, the term "labeling" refers to the
attachment of a chemical moiety such as a fluorochrome to
a protein. The attachment is typically through a covalent
25 bond but may also include non-covalent interactions as
well. Therefore, the term "labeled proteins" refers to
proteins which have chemical moieties such as fluorochromes
attached to them.

The invention provides a composition of matter,
30 comprising $(S_1 \kappa A)_{n1} (S_2 \kappa D)_{n2}$ wherein S_1 and S_2 are two proteins
which are associated in one state and substantially
disassociated in another state, and D and A are different
fluorochromes, the emission wavelength of one fluorochrome

overlapping the excitation wavelength of the other
fluorochrome, the distance between D and A is in
sufficiently close proximity to allow the radiationless
transfer of energy between the fluorochromes, and n1 and n2
5 representing the stoichiometry of said proteins, and
wherein S_1 or S_2 is capable of binding an analyte X, the
binding of X affects the affinity of S_1 and S_2 for each
other.

The two proteins S_1 and S_2 which are labeled with
10 fluorochromes are typically subunits of an enzyme but may
include any two proteins which exhibit associated and
substantially disassociated states. Examples of such
protein pairs include the subunits for cAMP dependent
protein kinase (cAMPdPK), the subunits of GTP-binding
15 proteins, calmodulin and calmodulin dependent protein
kinases and the regulatory and catalytic domains of protein
kinase C.

When in the associated state, the subunits form a
protein complex such as a holoenzyme. The number of
20 proteins within the complex can vary depending on the
stoichiometry between S_1 and S_2. Additionally, the complex
may include proteins other than S_1 and S_2. These other
proteins within the complex may be considered as components
of S_1 or S_2 so long as they maintain a stable association
25 with either S_1 or S_2.

When in the disassociated state, at least one of the
proteins will have substantially changed its distance from
the other protein. For example, the disassociated state
for cAMPdPK consists of free catalytic and regulatory
30 proteins or subunits. Likewise, GTP-binding proteins are
disassociated into alpha and beta-gamma subunits,
calmodulin dependent protein kinases disassociate from
calmodulin, and protein kinase C regulatory and catalytic
domains initially move apart and are subsequently separated

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by proteolysis.

Radiationless energy transfer is based on the
biophysical properties of fluorochromes. These principles
are reviewed in Lakowicz, J., 1983, Principles of
5   Fluorescence Spectroscopy, Plenum Press, New York and Jovin
and Jovin, 1989, in Cell Structure and Function by
Microspectrofluorometry, edited by E. Kohen and J. G.
Hirschberg, Academic Press, both of which are incorporated
herein by reference. Briefly, a fluorochrome absorbs light
10   energy at a characteristic wavelength. This wavelength is
also known as the excitation wavelength. The energy
absorbed by a fluorochrome is subsequently released through
various pathways, one being emission of photons to produce
fluorescence. The wavelength of light being emitted is
15   known as the emission wavelength and is an inherent
characteristic of a particular fluorochrome. Radiationless
energy transfer is the quantum-mechanical process by which
the energy of the excited state of one fluorochrome is
transferred without actual photon emission to a second
20   fluorochrome. That energy may then be subsequently
released at the emission wavelength of the second
fluorochrome. The first fluorochrome is generally termed
the donor (D) and has an excited state of higher energy
than that of the second fluorochrome, termed the acceptor
25   (A). The essential features of the process are that the
emission spectrum of the donor overlap with the excitation
spectrum of the acceptor, and that the donor and acceptor
be sufficiently close. The distance over which
radiationless energy transfer is effective depends on many
30   factors including the fluorescence quantum efficiency of
the donor, the extinction coefficient of the acceptor, the
degree of overlap of their respective spectra, the
refractive index of the medium, and the relative
orientation of the transition moments of the two
35   fluorochromes, but is typically 4-6 nm for favorable pairs
of donor and acceptor. Beyond the optimum range of

intermolecular distances, the energy transfer efficiency falls off as the inverse sixth power of the distance.

The analyte X capable of being bound by either S_1 or S_2, can be, for example, a second messenger such as cAMP,
5   calcium, cGMP, and diacylglycerol; relevant hormone-receptor complexes; or an organic molecule such as phorbol ester. When bound to such molecules, S_1 and S_2 may be in either the associated or disassociated states.

The invention provides a composition of matter wherein
10 S_1 and S_2 are proteins, or subunits of a complex, such as a holoenzyme. Such holoenzymes can be, for example, cAMPdPK, GTP-binding proteins, calmodulin dependent protein kinase and protein kinase C.

In one embodiment, the invention also provides a
15 composition of matter wherein S_1 is the regulatory subunit of cAMPdPK and S_2 is the catalytic subunit of cAMPdPK and are labeled with different fluorochromes such that the emission wavelength of one overlaps the excitation wavelength of the other.

20   For example, in the absence of cAMP, the regulatory (R) and catalytic (C) subunits of cAMPdPK have a stoichiometry of R_2C_2 as shown in figure 1. The complex is inactive as a kinase, since the R subunits inhibit the kinase domains of the C subunits. Upon the binding of cAMP
25 by R, the R subunits dissociate from the C subunits, releasing the biological activity of the latter. The invention includes labeling the R subunits with one fluorochrome and the C subunits with a different fluorochrome, such that in the intact R_2C_2 complex, light
30 energy absorbed by one fluorochrome is transferred to the other fluorochrome by the process of radiationless energy transfer. This phenomenon requires that the wavelengths of fluorescent emission from one fluorochrome, the donor (D),

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overlap the wavelengths of excitation of the other
fluorochrome, the acceptor (A), and that the D and A
moieties be in sufficiently close proximity to allow the
radiationless transfer of energy between the fluorochrome
5 pairs.   In   the   case   of   fluorescein   and
tetramethylrhodamine, this distance is less than about 4 to
6 nm. For other fluorochrome pairs, the distance can be
determined by those skilled in the art. Provided suitable
choices are made for D and A, this condition of close
10 proximity is reasonably well satisfied in a holoenzyme
complex such as that for cAMPdPK, so that energy transfer
occurs with considerable efficiency.

      The transferred energy is easily detectable by
excitation at wavelengths absorbed by D, since emission
15 from D is quenched and the energy appears instead at longer
wavelengths characteristic of A's emission.

      Subunits $R_K D$ and $C_K A$ can be paired to make
$(R_K D)_2 (C_K A)_2$, or $R_K A$ and $C_K D$ can be combined into
$(R_K A)_2 (C_K D)_2$, where K denotes attachment of the indicated
20 fluorochrome. Once cAMP has dissociated R from C, D and A
thereby become widely separated from each other and unable
to transfer energy from D to A. With the subunits
dissociated, illumination at wavelengths absorbed by D
simply causes fluorescence emission from D, with no
25 involvement of A, so that the composite emission spectrum
shifts towards the shorter wavelengths of D.

      Holoenzyme labeled with two different fluorochromes as
described above is found to have essentially identical
sensitivity to cAMP as the native holoenzyme. This
30 property allows the use of protein complexes such as
cAMPdPK to be used as measuring devices or sensors for a
variety of biological molecules. Moreover, it guarantees
that the sensor has the optimal affinity for such
biological molecules as cAMP, for example, since native

holoenzyme is the main endogenous sensor for cAMP and the
concentrations of interest are precisely those which affect
this enzyme. Moreover, the activity of labeled enzyme as
a kinase upon stimulation by cAMP is found to be identical

5   to native holoenzyme likewise stimulated. This property is
valuable because it minimizes any perturbation of the
biological effectiveness of cAMP within cells into which
the sensor has been introduced. If the sensor were not an
effective kinase, then any cAMP bound to it would be

10   prevented from exerting biological activity so that the
sensor would inherently inhibit the signaling pathway whose
activity was to be measured. However, if the sensor is a
normal kinase, cAMP molecules bound to it are biologically
fully effective and not removed from mediating such events.

15   The invention also provides a method of determining
the concentration of an analyte in a sample comprising the
steps of: (a) labeling proteins S_1 and S_2 with
fluorochromes A and D, where S_1 or S_2 is capable of binding
the analyte and S_1 and S_2 are associated in one state and

20   substantially   disassociated   in   another   state,   the
equilibrium of which is controlled by the concentration of
free analyte, the fluorochromes are selected such that the
emission wavelength of one fluorochrome overlaps the
excitation wavelength of the other fluorochrome to form

25   labeled   proteins $(S_1 D)_{n1}$ $(S_2 A)_{n2}$ or $(S_1 A)_{n1}$ $(S_2 D)_{n2}$;   (b)
contacting the labeled proteins with the sample; (c)
providing energy near the excitation wavelength of D or A;
(d) measuring the fluorescences emitted at the emission
wavelengths of A and D respectively, and determining the

30   extent of energy transfer from D to A by the ratio of their
emission amplitudes, or by the fluorescence lifetime of D,
or by the rate of photobleaching of D.   Any of these
parameters, but most conveniently the ratio of emission
amplitudes, can be related to the analyte concentration by

35   a calibration curve established with samples of known
analyte concentration.

In one embodiment, holoenzyme labeled with fluorescein
on the C subunit and tetramethylrhodamine on the R subunit,
for example, can be used to measure the concentration of
cAMP _in vitro_ and _in vivo_. For the former, the enzyme is
5   titrated with cAMP while measuring its fluorescent emission
following excitation at 490 to 500 nm. A plot of the
emission ratio (520:580 nm are the optimal wavelengths)
against known concentrations of cAMP can be used as a
standard curve for determining cAMP concentrations in
10 unknown solutions under the same conditions. To measure _in
vivo_ cAMP concentrations inside living cells requires
incorporation of the labeled holoenzyme into the cells of
interest by microinjection, electroporation, lipofection,
bead loading, scrape loading or any other comparable and
15 well-known technique. Such methods are described in
Riabowol et al., (1988) Cold Spring Harbor Symposia on
Quantitative Biology 53:85-90; Sambrook et al., Molecular
Cloning: A Laboratory Manual, (1989) Cold Spring Harbor
Laboratory and Ausubel et al., Current Protocols in
20 Molecular Biology, (1987), John Wiley and Sons, all of
which are incorporated herein by reference. Excitation at
490 to 500 nm on a fluorescence microscope and detection of
the emission near the two optimal wavelengths (520 and 580
nm) is achieved by a photometer or television camera.
25 Calibration of the minimum and maximum ratio value is
achieved by first setting the zero cAMP concentration value
by addition of a cAMP antagonist, adenosine -3', 5'- cyclic
monophosphothioate, Rp-isomer (Rp-cAMPS) which inhibits
dissociation of the holoenzyme. Subsequent removal of the
30 cAMP antagonist and addition of the membrane-permeable cAMP
analog gives complete dissociation and hence intermediate
cAMP values can be determined using the standard curve
derived _in vitro_.

Currently preferred choices of fluorochromes are
35 fluorescein for the donor dye, which has emission
wavelengths of about 510 to 550 nm, and

tetramethylrhodamine for the acceptor, which has excitation
wavelengths of about 510 to 550 nm, both being attached to
the subunits via isothiocyanates under conditions to favor
reaction with lysines. However, a variety of fluorochromes
5   and derivatives thereof can be used so long as the emission
wavelength of fluorochrome D overlaps the excitation
wavelength of fluorochrome A and results in energy transfer
from D to A. For example, D and A fluorochrome pairs may
be a 7 aminocoumarin and dipyrromethene-borate, an
10   indocarbocyanine and rhodamine X or a phycoerythrin and
allophycocyanin.

The invention also provides for a method of preparing
a complex comprising protein subunits S_1 and S_2 which are
associated in one state and substantially disassociated in
15   another state, the equilibrium between which is controlled
by the concentration of a free analyte X, and wherein S_1 or
S_2 is capable of binding the analyte, comprising: (a)
attaching fluorochrome A to S_1, said fluorochrome A having
an excitation wavelength which overlaps with the emission
20   wavelength of a second fluorochrome D; (b) attaching the
second fluorochrome D to S_2; (c) mixing the labeled subunits
so that a complex is formed wherein the fluorochromes are
in sufficiently close proximity to allow radiationless
transfer of energy between the fluorochromes; and (d)
25   recovering the labeled subunits as a complex.

In one embodiment, R and C are separately available as
recombinant proteins, for example from expression in E.
coli, which are then labeled. However, careful adjustment
of reaction conditions is necessary so that the act of
30   labeling does not prevent reassociation into holoenzyme or
subsequent cAMP sensitivity and kinase activity. Previous
literature has suggested that labeling isolated C with
fluorescein isothiocyanate ruined its ability to combine
with R; under milder conditions, however, C was not damaged
35   as described below.

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The invention additionally provides a method of preparing a complex comprising protein subunits S_1 and S_2 which are associated in one state and substantially disassociated in another state, the equilibrium between which is controlled by the concentration of a free analyte
5 X, and wherein S_1 or S_2 is capable of binding the analyte, comprising: (a) attaching fluorochrome A to a complex comprising S_1 and S_2, said fluorochrome A having an excitation wavelength which overlaps with the emission
10 wavelength of a second fluorochrome D; (b) attaching the second fluorochrome D to a second complex comprising S_1 and S_2; (c) separating the subunits of said first and second complex to obtain labeled subunits $S_1\kappa D$, $S_2\kappa D$, $S_1\kappa A$ and $S_2\kappa A$; and (d) mixing the labeled subunits wherein said
15 fluorochromes are in sufficiently close proximity to allow radiationless transfer of energy between the fluorochromes to form label complexes $(S_1\kappa D)_{n1} (S_2\kappa A)_{n2}$ or $(S_1\kappa A)_{n1} (S_2\kappa D)_{n2}$, wherein n1 and n2 represent the stoichiometry of the labeled subunits.

20   An alternative method is to label pre-formed holoenzyme R_2C_2 with D, thereby preparing $(R\kappa D)_2(C\kappa D)_2$ in which the sites of interaction between R and C are inherently protected. A separate batch of R_2C_2 is analogously labeled with A to make $(R\kappa A)_2(C\kappa A)_2$. Then
25 labeled R and labeled C are dissociated and separated from each other by passage through a cAMP affinity column, to which labeled R sticks while labeled C passes through. R can be recovered from the column by elution with free cAMP. When this separation is applied to each batch, one obtains
30 $R\kappa D$, $C\kappa D$, $R\kappa A$, and $C\kappa A$, which are then recombined in different pairings to make $(R\kappa D)_2(C\kappa A)_2$ as one preparation and $(R\kappa A)_2(C\kappa D)_2$ as another.

The holoenzyme or individual subunits of cAMPdPK can be prepared from mammalian sources such as that described
35 by Bubis et al., (1985) Biochemistry, 24:2163-2170, and

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Zick et al., J. Biol. Chem., (1982) 257:2287-2293, both of
which are herein incorporated by reference. If subunits
from recombinant or mammalian sources are used for the
preparation of holoenzyme then reconstitution can be
5   performed as described in the Examples.

The following examples are intended to illustrate but
not limit the invention.

EXAMPLE I
Recombinant Expression and Purification
10   of cAMP-Dependent Protein Kinase Regulator Subunit

Reagents were purchased from the following companies:
histone IIA, phenylmethylsulfonyl fluoride, isopropyl-β-D-
thiogalactopyranoside (IPTG), and bovine serum albumin,
(Sigma, St. Louis, MO); N^{α}-p-tosyl-L-lysine chloromethyl
15 ketone and cAMP, (United States Biochemical Corp.,
Cleveland, OH); 2,8-[^{3}H]cAMP (27 Ci/mmol) and 8-N_3[^{32}P]cAMP
(20-30 Ci/mmol), (ICN); Sephadex G-150 and cAMP-agarose (N^6-
ethane spacer), (Pharmacia, Pleasant Hill, CA);
nitrocellulose (0.45 μm) and electrophoresis reagents,
20 (Bio-Rad, Richmond, CA); and Cytoscint, (West Chem).
Enzymes used in DNA manipulations were obtained from either
Bethesda Research Laboratories (Gaithersberg, MD) or
Boehringer Mannheim (Indianapolis, IN) and were used
according to the manufacturer's specifications.
25 Fluorescent labels were obtained from Molecular Probes
(Eugene, OR) and cAMP analogs were obtained from Biolog (La
Jolla, CA). All other reagents were analytical grade.

Plasmid 62C12, Lee et al., (1983) Proc. Natl. Acad.
Sci. U.S.A., 80:3608-3612, and herein incorporated by
30 reference, which contains the full-length cDNA of bovine R[1]
in pBR322 was subcloned into pUC7, Viera et al., (1982)
Gene, 19:259-268, and herein incorporated by reference for
cDNA expression.

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For subcloning, DNA manipulations were carried out according to Maniatis et al., (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, and herein incorporated by reference.

5   Briefly, 62C12 was digested with NarI, and the 3.6-kilobase (kb) fragment containing the cDNA was isolated. Following partial digestion with NcoI, the two large fragments, the full-length cDNA fragment (1155) base pairs (bp), and a truncated fragment (1020 base pairs) were isolated as a

10   mixture, filled-in with Klenow fragment, and ligated into pUC7 that had been linearized with Hinc II. The 135-base pair fragment also was isolated, radiolabeled with [gamma-^{32}P]ATP, and used to identify those clones which contained the full-length insert. Twenty colonies were selected, and

15   the DNA from each was prepared and digested with Bg/I in order to establish the orientation of the insert. The DNA from three clones gave a restriction pattern which indicated that the R^1 coding segment was inserted in the correct orientation with regard to the lac Z' gene. These

20   clones were further characterized for expression of the protein.

Since basal levels of cAMP are high in E. coli, the following method was devised for the accurate detection of R in crude extracts independent of exogenous cAMP. This

25   method could be used for rapid screening. Protein samples were electrophoresed on 12.5% polyacrylamide gels (1.5 mm) containing sodium dodecyl sulfate according to the method of Laemmli (1970) Nature 227:680-685. Proteins were then transferred from the gels to nitrocellulose filters using

30   an electroblotting apparatus (Hoefer Scientific Instruments). Gels were electrotransferred for 4 to 6 hours at 500 mA in 20 mM Tris, 154 mM glycine, 20% methanol (pH 8.3). The nitrocellulose was incubated for 1 hour at room temperature with 0.05% Tween 20 in 150 mM sodium

35   chloride and 10 mM Tris (pH 7.5) and then washed at room temperature with the same buffer except that Tween 20 was

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omitted. The filter was incubated with $8-N_3[^{32}P]cAMP$ (20 nM) in the same buffer for 30 minutes in the dark at room temperature. After washing with ice-cold buffer, the filter was irradiated with UV S-11 lamp (254 nm) for 5

5 minutes, washed again, blotted dry, and subjected to autoradiography. The R-subunit also was detected by Millipore filtration using $2,8-[^3H]cAMP$ as described by Kerlavage et al., (1982) J. Biol. Chem., 257:1749-1754, and herein incorporated by reference.

10   Purification of the regulatory subunit was performed by inoculating 5 mls of L-broth containing ampicillin (50 µg/ml) with the highest expressing transformant and growing at 37°C until the cell density reached OD_{550} = 0.1-0.5. The culture was then transferred to 1 liter of L-broth

15 containing ampicillin and incubated (12 to 16 hours). Unless indicated, all subsequent procedures were carried out at 4°C. the cells were centrifuged at 5,000 x g for 30 minutes, and the resulting pellet was resuspended in buffer I (20 mM potassium phosphate, 5 mM EDTA, 5 mM 2-

20 mercaptoethanol, 15 mg/liter N^{α}-p-tosyl-L-lysine chloromethyl ketone, and 15 mg/liter phenylmethylsulfonyl fluoride). This suspension was passed through a French pressure cell twice and then centrifuged at 5,000 x g for 20 minutes. The pellet was re-extracted with the same

25 buffer. Ammonium sulfate was added slowly to the pooled supernatant fractions until a concentration of 70% was reached. After 1 hour, the precipitated protein was recovered by centrifugation at 12,000 x g for 30 minutes, redissolved in buffer I, and dialyzed against the same

30 buffer overnight. Fractions were assayed for $[^3H]cAMP$ binding and analyzed by polyacrylamide gel electrophoresis followed by photolabeling as described above. The dialyzed sample was then added to cAMP-agarose and gently rotated overnight. After removing the supernatant solution, the

35 resin was washed with buffer I containing 2 M NaCl until the absorbance at 280 nm reached 0 and then washed again

with buffer I. The R-subunit was eluted with 2 volumes of
cAMP (50 mM) in buffer I at 30°C for 1 hour. The elution
was repeated at least once.

     The eluate from the cAMP-agarose resin frequently
5 contained some proteolytic degradation products of R in
addition to full-length R, which was separated on a 70 cm
Sephadex G-150 column equilibrated and eluted with 25 mM
potassium phosphate, 5 mM 2-mercaptoethanol, 5 mM EDTA, and
150 mM NaCl (pH 6.5). Fractions (2 ml) were collected at
10 a flow rate of 7 ml/hour. The R-subunit eluted close to
the void volume and was well-resolved from the 35-kDa
proteolytic fragment.

     The recombinant cAMP dependent regulatory subunit was
sequenced on an Applied Biosystems Gas-phase Sequencer.
15 Two nanomoles of R-subunit were sequenced, and the
phenylthiohydantoin derivatives were analyzed on a Beckman
HPLC system using a cyano column (IBM) according to the
procedure of Hunkapillar and Hood, (1983) Methods Enzymol.,
91:486-493, and herein incorporated by reference.

20   EXAMPLE II
                   Recombinant Expression and Purification of
                  cAMP-Dependent Protein Kinase Catalytic Subunit

     Plasmid containing the full length clone for the α
catalytic subunit of murine cAMP-dependent protein kinase,
25 pMCO, Uhlen et al., (1986) Proc. Natl. Acad. Sci. USA,
83:1300-1304, which is incorporated herein by reference,
was subcloned into M13 mp18 for mutagenesis. The bacterial
expression vector, pT7-7, Tabor and Richardson, (1985)
Proc. Natl. Acad. Sci. USA, 82:1074-1078, and herein
30 incorporated by reference was used for recombinant
expression. The E. coli strains used to express the
catalytic subunit were: JM101, 222, K38 and BL21 (DE3).

Before introducing a unique NdeI restriction site into the catalytic subunit gene at the initiation methionine, the 1.9-kilobase SacI fragment from pMCO containing the coding region for the catalytic subunit was excised and
5   religated into the SacI site of M13mp18. Single stranded DNA was prepared and used as template for oligonucleotide-directed mutagenesis with a 24-base oligonucleotide (5'-CACGCCGCCCATATGGGCAACGCC-3') according to the procedure described by Zoller and Smith, (1984) DNA 3:479-488, and
10 herein incorporated by reference. The mutation was verified by dideoxy sequencing according to Sanger et al., (1980) J. Mol. Biol. 143:161-178, and herein incorporated by reference and by mapping with restriction enzymes.

Construction of pLWS-3 was performed by excising a
15 1988-base fragment from the M13 construct described above with NdeI and HindIII, purified from an agar gel, and ligated into the NdeI-HindIII restriction sites of pT7-7. This ligation mixture was used to transform E. coli JM101 using the procedure described by Maniatis et al., ibid.

20   Transformants were selected and screened for plasmid DNA containing the cDNA insert following digestion with NdeI and HindIII. DNA was isolated from this plasmid, pLWS-3, and the sequence confirmed by mapping with restriction enzymes and by sequence analysis. The plasmid
25 was then used to transform E. coli BL21(DE3), and transformants were screened for expression of the catalytic subunit by monitoring for the appearance of a 38-40 kDa band following polyacrylamide gel electrophoresis.

For expression of the catalytic subunit, one liter of
30 L-broth (10% Tryptone, 5% yeast extract, and 5% NaCl) containing ampicillin was inoculated with 5 ml of a stationary phase culture of BL21(DE3) transformed with pLWS-3 and grown at 37°C with vigorous shaking until an absorbance of 0.5 at 590 nm was reached. After adding

isopropyl-ß-D-thiogalactopyranoside   to   a   final
concentration of 0.5 mM, the cells were grown for an
additional 3 hours at 37°C and then harvested by
centrifugation.

5   Purification of the recombinant catalytic subunit was
performed by resuspending the pellet in 6 ml of lysis
buffer (30 mM MES, pH 6.45, 50 mM KCl, 1 mM EDTA, and 5 mM
ß-mercaptoethanol) at 4°C and passed twice through a French
pressure cell. Additional lysis buffer was added to bring
10   the total volume to 30 ml. After centrifugation at 12,000
x g for 15 minutes at 4°C, the supernatant was removed and
diluted with ice-cold water until the conductivity was
below 1.3 mmhos. The diluted supernatant was applied to a
phosphocellulose column (Whatman P11, 2.0 x 8.0 cm) pre-
15   equilibrated with buffer I (30 mM MES, pH 6.45, 1 mM EDTA).
After washing with the equilibration buffer until the
absorbance at 280 nm was 0, the catalytic subunit was
eluted with a linear gradient of buffer I (50 ml) to 0.5 M
potassium phosphate, pH 7.0 (50 ml). Two-ml fractions were
20   collected, and those containing kinase activity were
pooled.   The protein was precipitated by the batchwise
addition of solid NH_4SO_4, with constant stirring, until the
solution was 80% saturated. The precipitate was collected
by centrifugation at 12,000 x g for 20 minutes and then
25   resuspended in 0.8 ml of H_2O.   After removing the
undissolved material by centrifugation, the protein
solution was loaded onto an Ultrogel AcA 44 (LKB) gel
filtration column (1.0 x 116 cm) equilibrated with 25 mM
potassium phosphate, pH 6.8.   The fractions containing
30   kinase activity were combined and concentrated to 1 mg/ml
by ultrafiltration using an Amicon apparatus and a PM-30
membrane.   Based on polyacrylamide gel electrophoresis
carried out in the presence of sodium dodecyl sulfate (SDS)
according to Laemmli, (1970) Nature 227:680-685, which is
35   incorporated herein by reference, the recombinant catalytic
subunit was nearly homogeneous, and the yield was 3.5 to

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4.0 mg/liter of culture.

Recombinant catalytic subunit was diluted to 0.04
mg/ml with 20 mM Tris, pH 7.5. Fifty-μl aliquots were kept
on ice and then placed in a 49°C water bath. At the
5   indicated times, tubes were removed, placed on ice, and
assayed for stability of the recombinant protein.

Isoelectric focusing was carried out using a LKB
multiphor model 2117 with pre-cast LKB Ampholine Pagplates,
pH. 3.5 to 9.5. The standards were bovine carbonic
10 anhydrase (pI 6.57), myoglobin (pI 6.67 and 7.16), and
lactate dehydrogenase (pI 8.55).

The molecular weight of the recombinant catalytic
subunit was determined by gel filtration using a LKB
Ultrogel AcA 44 column (1 x 116 cm). The running buffer
15 was 25 mM potassium phosphate (pH 6.8), 200 mM KCl, and
0.2% sodium azide. The standards and their respective M
values were ribonuclease A (13,700), chymotrypsinogen A
(25,000), porcine catalytic subunit (38,000), ovalbumin
(43,000), and bovine serum albumin (67,000).

20   The recombinant catalytic subunit was sequenced on a
Applied Biosystems gas-phase sequencer as described in
Example I.

EXAMPLE III

Purification of Mammalian Catalytic Subunit

25   Porcine hearts (40 hearts) were homogenized and then
centrifuged. The supernatant (conductivity 3 mmho) was
passed directly through a DE-23 column (4 L resin)
equilibrated in 15 mM potassium phosphate, pH 6.5, 2 mM
EDTA, 5 mM ß-mercaptoethanol. The protein was eluted from
30 the resin with buffer E (40 mM potassium phosphate, pH 6.5,
2 mM EDTA, 5 mM ß-mercaptoethanol). The pooled kinase peak

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was immediately adjusted to a conductivity of 4 mmho at
which point 1.5 L of DE-52 resin was added and the whole
was mechanically stirred for 2 hours. The supernatant was
poured off and the resin was poured into a column and
5   washed extensively with buffer E, followed by extensive
washing with buffer F (17 mM potassium phosphate, pH 6.1,
2 mM EDTA, 5 mM β-mercaptoethanol). The C-subunit was then
eluted with 3 L of 220 μM cAMP followed by 8 L of buffer F
and the eluent was passed through a CM-Sepharose column (50
10 mL volume) and then eluted with a linear gradient of buffer
F containing 10% glycerol (250 mL) to 200 mM potassium
phosphate, pH 6.1, 10% glycerol, 2 mM EDTA, 5 mM β-
mercaptoethanol (250 mL).

EXAMPLE IV
15 Preparation of Labeled cAMP-Dependent Protein Kinase

    (a)   Labeling subunits:

    Recombinant   catalytic   and   regulatory   subunits
(approximately 0.5-2.0 mg/ml) of mammalian cAMP-dependent
protein kinase were purified as described in Examples I and
20 II and were dialyzed separately against 25 mM bicine pH
8.0, 0.1 mM EDTA for 4 to 5 hours at 0°C. The catalytic
subunit   was   labeled   with   0.3   mM   fluorescein   5'-
isothiocyanate (FITC; Molecular Probes, Eugene, OR) in the
presence of 8 mM $MgCl_2$ and 5 mM ATP to prevent inactivation
25 of kinase activity. The regulatory subunit was labeled
with 0.5 mM tetramethylrhodamine isothiocyanate (TRITC,
isomer G; Molecular Probes, Eugene, OR). Both labelings
were   allowed   to   proceed   from   30   minutes   at   room
temperature, then quenched by addition of 5 mM glycine for
30 10 to 15 minutes. The excess dye was removed by passing
each protein solution through a Sephadex G-25 column (3
ml), eluted with 25 mM potassium phosphate pH 6.8, 2 mM
EDTA, 5 mM 2-mercaptoethanol, 10% glycerol. The first
colored or fluorescent band was collected.

The FITC-labeled catalytic and TRITC-labeled regulatory subunits (equal amounts by weight) were then mixed and dialyzed against 25 mM potassium phosphate pH 6.8, 0.5 mM $MgCl_2$, 0.1 mM ATP, 5 mM 2-mercaptoethanol, 5%

5 glycerol for 3 to 5 days at 4°C. The formation of holoenzyme could be demonstrated by kinase assay measured using Kemptide as substrate (Slice & Taylor, ibid.).

(b) Labeling holoenzyme:

cAMP-dependent protein kinase holoenzyme (0.5 - 1.0

10 mg/ml) prepared from mammalian catalytic and recombinant regulatory subunits was dialyzed against 25 mM bicine pH 8.0, 0.1 mM EDTA at 4°C. $MgCl_2$ and ATP were added to give final concentrations of 8 mM and 5 mM respectively. The proteins were labeled with either fluorescein 5'-

15 isothiocyanate or tetramethylrhodamine isothiocyanate (Isomer G) at final concentrations of 0.1 - 0.5 mM at room temperature for 20 to 30 minutes, followed by quenching with 5 mM glycine pH 8.0 for 10 minutes. Excess free dye was (partially) removed by passing the protein solutions

20 through a column of Sephadex G-25 (3 ml), eluting with 10 mM potassium phosphate pH 6.7, 150 mM KCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA (Buffer I) and collecting the first colored fluorescent band.

The labeled holoenzymes were then passed through a

25 cAMP affinity column (0.25 ml) (Wolfgang, W. et al., FEBS Lett. 99, 62 (1979)) at a rate of approximately 0.1 ml/minute and then eluted with 2 column volumes of the Buffer I at 4°C. The collected fractions contained labeled catalytic subunit. The column was eluted with Buffer I

30 containing 1 M KCl (2 column volumes) and the Buffer I (2 column volumes) again. These washes may contain some additional labeled catalytic subunit. To recover the labeled regulatory subunit, the column was eluted slowly with 30 mM cAMP in Buffer I (4 column volumes) at room

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temperature over a 2 hour period.

The differently labeled subunits were recombined in appropriate donor-acceptor pairs and dialyzed against several changes of 25 mM potassium phosphate pH 6.8, 0.5 mM
5   $MgCl_2$, 0.1 mM ATP, 5 mM 2-mercaptoethanol, 5% glycerol for 3 to 5 days at 4°C. The formation of cAMPdPK holoenzyme could be monitored by loss of kinase activity, which can be recovered on addition of cyclic AMP.

EXAMPLE V
10   Titration of cAMP With Labeled Holoenzyme In Vitro

Holoenzyme (20 nM) labeled with 5'-fluorescein isothiocyanate (5'-FITC) on the catalytic subunit and tetramethylrhodamine isothiocyanate (isomer G) on the regulatory subunit, was dissolved in 135 mM KCl, 10 mM
15   KₖMOPS pH 7.2, 2 mM $MgCl_2$, 1 mM ATP at room temperature. The fluorescence emission was measured following excitation at 495 nm before and after addition of 20, 50, 100, 200, 400 nM, 1 and 51 μM cAMP (figure 2). Only the 0, 50, 200, 1000 nM cAMP spectra are shown in Figure 1 for clarity.
20   The calculated ratio of emission at 520 nm to 580 nm, normalized to the ratio at 0 cAMP is plotted with cAMP concentration in Figure 2 (inset), indicated a half-maximal change in ratio as about 100 nM cAMP.

EXAMPLE VI
25   In Vivo Measurement of cAMP
in Single Smooth Muscle Cells

Smooth muscle cells (BC_3H_1 cell line) were microinjected as described by Riabowol, Gilman & Feramisco, Cold Spring Harbor Symposia on Quantitative Biology, 53:85-
30   90 (1988), which is incorporated herein by reference, with 60 μM holoenzyme that was labeled with 5'FITC on the C subunit and TRITC on the R^I subunit (figure 3). After 15 to

20 minutes, the cells were illuminated on a fluorescence microscope stage with 490 nm light at room temperature. The emitted light was detected by a Silicone Intensified Target camera (Dage-NTI) after passing through either a

5　500-530 nm bandpass filter or a 580 nm longpass filter. The two pictures per time point were digitised by an image processor, the ratio of 500-530 nm to > 580 nm calculated at each pixel and displayed in pseudocolors on a television monitor. The average ratio in a representative cell was

10　determined at each time point to give Figure 3. At time point 1, 250 μM dibutyryl cAMP, a membrane-permeable cAMP analog, was added resulting in a gradual increase in ratio indicating a rise in cAMP analog inside the cell.

　　　In a separate experiment BC_3H_1 cells were microinjected

15　and the emission ratio determined as described above. The following additions were made and are shown in figure 4 (1) 100 nM isoproterenol (2) 100 nM propranolol (3) 50 μM forskolin (4) 250 μM dibutyryl cAMP. Addition of isoproterenol, a β_2-adrenergic agonist elicited a rapid rise

20　in emission ratio and therefore cAMP concentration within the cell, which was subsequently decreased by propranolol (a β_2-adrenergic antagonist). Direct activation of adenylate cyclase by forskolin caused a maximal emission ratio as no further effect was seen with dibutyryl cAMP, a

25　membrane-permeable cAMP analog.

　　　Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention.

30　Accordingly, the invention is limited only by the following claims.

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WE CLAIM:

1. A composition of matter, comprising $(S_1\kappa A)_{n1}$ $(S_2\kappa D)_{n2}$ wherein S_1 and S_2 are two proteins which are associated in one state, and substantially disassociated in another state the equilibrium between which is controlled
5   by the concentration of a free analyte X, and D and A are different fluorochromes, the emission wavelength of one fluorochrome overlapping the excitation wavelength of the other fluorochrome, the distance between D and A being in sufficiently close proximity to allow radiationless
10 transfer of energy between the fluorochromes, and n1 and n2 representing the stoichiometry of said proteins, and wherein S_1 or S_2 is capable of binding the analyte.

2. The composition of matter of claim 1, wherein S_1 and S_2 are subunits of an enzyme.

3. The composition of matter of claim 2, wherein the subunits are selected from the group of protein pairs consisting of the subunits for cAMPdPK, the subunits of GTP-binding protein subunits, calmodulin and calmodulin
5   dependent protein kinases and the regulatory and catalytic domains of protein kinase C.

4. The composition of matter of claim 1, wherein S_1 is the regulatory subunit of cAMPdPK and S_2 is the catalytic subunit of cAMPdPK.

5. The composition of matter of claim 1, wherein D and A are selected from the group consisting of fluorescein and tetramethylrhodamine, a 7 aminocoumarin and dipyrromethene-borate, an indocarbocyanine and rhodamine X and a phycoerythrin and allophycocyanin, or derivatives thereof, provided that the selected pair exhibit fluorescence energy transfer.

6. The composition of matter of claim 1, wherein the emission wavelength of D and the excitation wavelength of A are about 510 to 550 nm.

7. The composition of matter of claim 5 or 6, wherein the fluorochromes are fluorescein and tetramethylrhodamine or derivatives thereof.

8. The composition of matter of claim 4, wherein n1 is 2 and n2 is 2.

9. The composition of matter of claim 1, wherein the molecule capable of being bound by S_1 or S_2 is selected from the group consisting of cAMP, GTP, hormone receptor complexes, calcium, diacylglycerol and phorbol esters.

10. The composition of matter of claim 1, wherein the analyte is cAMP.

11. The composition of matter of claim 1, wherein S_1 and S_2 are recombinant proteins.

12. The composition of matter of claim 1, wherein one protein is a recombinant protein and one protein is derived from mammalian sources.

13. The composition of matter of claim 1, wherein S_1 and S_2 are derived from mammalian sources.

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14. A method of determining the concentration of
an analyte in a sample comprising the steps of:

(a) labeling proteins S_1 and S_2 with
     fluorochromes A and D, where S_1 or S_2

5   is capable of binding the analyte and
          S_1 and S_2 are associated in one state
          and substantially disassociated in
          another state, the equilibrium of which
          is controlled by the concentration of

10   free analyte, the fluorochromes
          characterized in that the emission
          wavelength of fluorochrome D overlaps
          the excitation wavelength of
          fluorochrome A to form labeled proteins

15        $(S_1\kappa D)_{n1}$ $(S_1\kappa A)_{n2}$;

(b) contacting the labeled proteins with
     the sample;

(c) providing energy near the excitation
     wavelength of D or A; and

20   (d) measuring the fluorescence emitted at
          the emission wavelength of A, the
          extent of energy transfer from D to A
          being determined by the emission
          amplitude.

25   15.   The method of claim 14, wherein the
measuring step further comprises measuring the fluorescence
emitted at the emission wavelength of the extent of energy
transfer from D to A being determined by the ratio of the
emission amplitudes of A and D.

30   16.   The method of claim 14, wherein the
measuring further step comprises measuring the fluorescence
lifetime of D or the rate of photobleaching of D.

          17.   The method of claim 14, wherein the sample
comprises an intact cell.

18. The method of claim 17, wherein the contacting step further comprises a method selected from the group consisting of microinjection, electroporation, lipofection, bead loading and scrape loading.

5   19. The method of claim 14, wherein the proteins S_1 and S_2 comprise subunits of an enzyme.

20. The method of claim 19, wherein the subunits are selected from the group of protein pairs consisting of the subunits for cAMPdPk, the subunits of GTP-binding 10   protein subunits, calmodulin and calmodulin dependent protein kinases and the regulatory and catalytic domains of protein kinase C.

21. The method of claim 14, wherein S_1 is the regulatory subunit of cAMPdPK and S_2 is the catalytic 15   subunit of cAMPdPK.

22. The method of claim 14, wherein D and A are selected from the group consisting of fluorescein and tetramethylrhodamine, a 7 aminocoumarin and dipyrromethene-borate, an indocarbocyanine and rhodamine X and a 20   phycoerythrin and allophycocyanin, or derivatives thereof, provided that the selected pair exhibit fluorescence energy transfer.

23. The method of claim 14, wherein the emission wavelength of D and the excitation wavelength of A are 25   about 510 to 550 nm.

24. The method of claim 22 or 23, wherein the fluorochromes are fluorescein and tetramethylrhodamine or derivatives thereof.

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25.   The method of claim 21, wherein n1 is 2 and
n2 is 2.

26.   The method of claim 14, wherein the analyte
is selected from the group consisting of cAMP, GTP, hormone
5   receptor complexes, calcium, diacylglycerol and phorbol
esters.

27.   The method of claim 14, wherein the analyte
is cAMP.

28.   The method of claim 14, wherein S_1 and S_2 are
10   recombinant proteins.

29.   The method of claim 14, wherein one protein
is a recombinant protein and one protein is derived from
mammalian sources.

30.   The method of claim 14, wherein S_1 and S_2 are
15   derived from mammalian sources.

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31. A method of preparing a complex comprising protein subunits S_1 and S_2 which are associated in one state and substantially disassociated in another state, the equilibrium between which is controlled by the

5 concentration of a free analyte X, and wherein S_1 or S_2 is capable of binding the analyte, comprising:

    (a) attaching fluorochrome A to S_1, said fluorochrome A having an excitation wavelength which overlaps with the

10   emission wavelength of a second fluorochrome D;

    (b) attaching the second fluorochrome D to S_2;

    (c) mixing the labeled subunits so that a

15   complex is formed wherein said fluorochromes are in sufficiently close proximity to allow radiationless transfer of energy between the fluorochromes; and

20   (d) recovering the labeled subunits as a complex.

32. The method of claim 31, wherein S_1 is the regulatory subunit of cAMPdPK and S_2 is the catalytic subunit of cAMPdPK.

33. The method of claim 31, wherein the fluorochromes are fluorescein tetramethylrhodamine or derivatives thereof.

34. The method of claim 31, wherein the analyte is cAMP.

35. The method of claim 31, wherein S_1 and S_2 are recombinant proteins.

36. The method of claim 31, wherein one protein is a recombinant protein and one protein is derived from mammalian sources.

37. The method of claim 31, wherein S_1 and S_2 are derived from mammalian sources.

38. A method of preparing a complex comprising protein subunits S_1 and S_2 which are associated in one state and substantially disassociated in another state, the equilibrium between which is controlled by the

5   concentration of a free analyte X, and wherein S_1 or S_2 is capable of binding the analyte, comprising:

(a) attaching fluorochrome A to a complex comprising S_1 and S_2, said fluorochrome A having an excitation wavelength which overlaps with the emission wavelength

10   of a second fluorochrome D;

(b) attaching the second fluorochrome D to a second complex comprising S_1 and S_2;

(c) separating the subunits of said first

15   and second complex to obtain labeled subunits $S_1 \kappa D$, $S_2 \kappa D$, $S_1 \kappa A$ and $S_2 \kappa A$; and

(d) mixing the labeled subunits wherein said fluorochromes are in sufficiently close proximity to allow radiationless

20   transfer of energy between the fluorochromes to form label complexes $(S_1 \kappa D)_{n1}$ $(S_2 \kappa A)_{n2}$ or $(S_1 \kappa A)_{n1}$ $(S_2 \kappa D)_{n2}$, wherein n1 and n2 represent the stoichiometry of the labeled subunits.

39. The method of claim 38, wherein S_1 is the regulatory subunit of cAMPdPK and S_2 is the catalytic subunit of cAMPdPK.

40. The method of claim 39, wherein S_2 is the regulatory subunit of cAMPdPK and S_1 is the catalytic subunit of cAMPdPK.

41. The method of claim 38, wherein the fluorochromes are fluorescein tetramethylrhodamine or derivatives thereof.

42. The method of claim 38, wherein the analyte is cAMP.

43. The method of claim 38, wherein S_1 and S_2 are recombinant proteins.

44. The method of claim 38, wherein one protein is a recombinant protein and one protein is derived from mammalian sources.

45. The method of claim 38, wherein S_1 and S_2 are derived from mammalian sources.
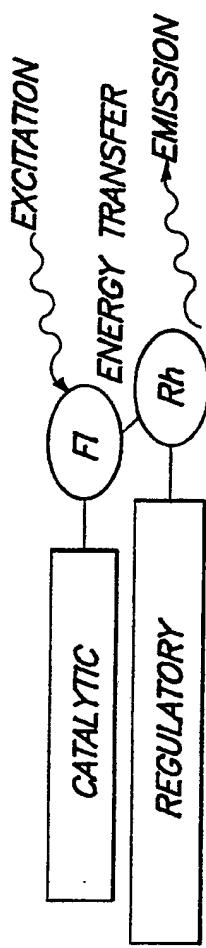
FIG. 1
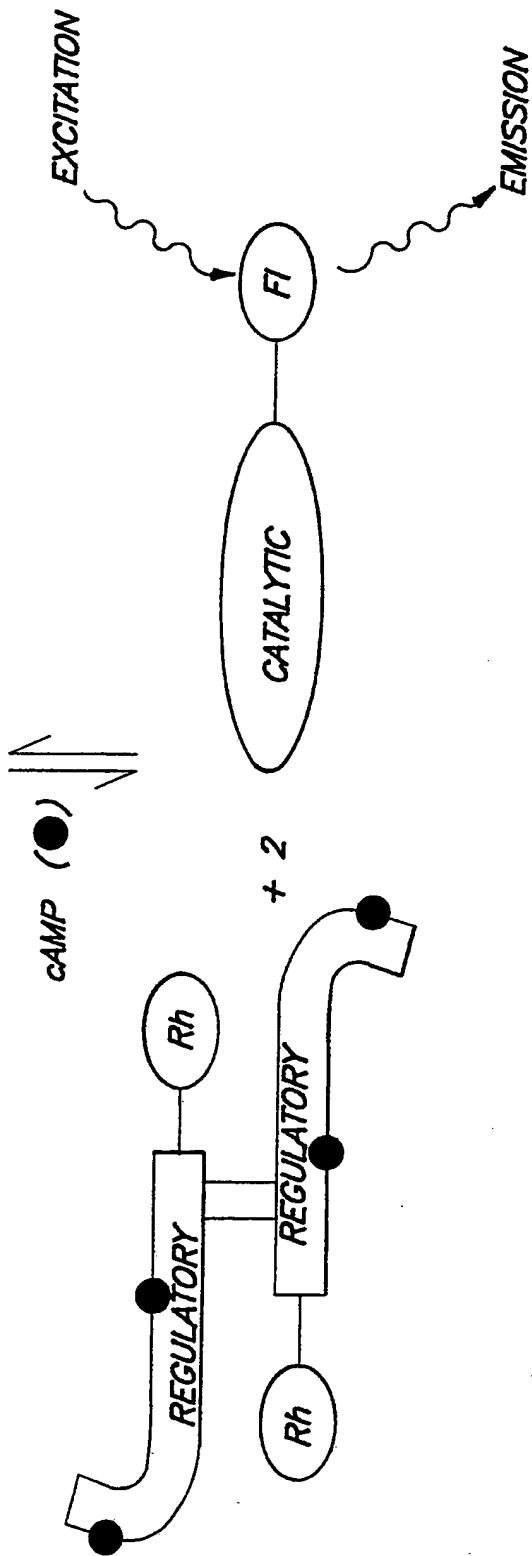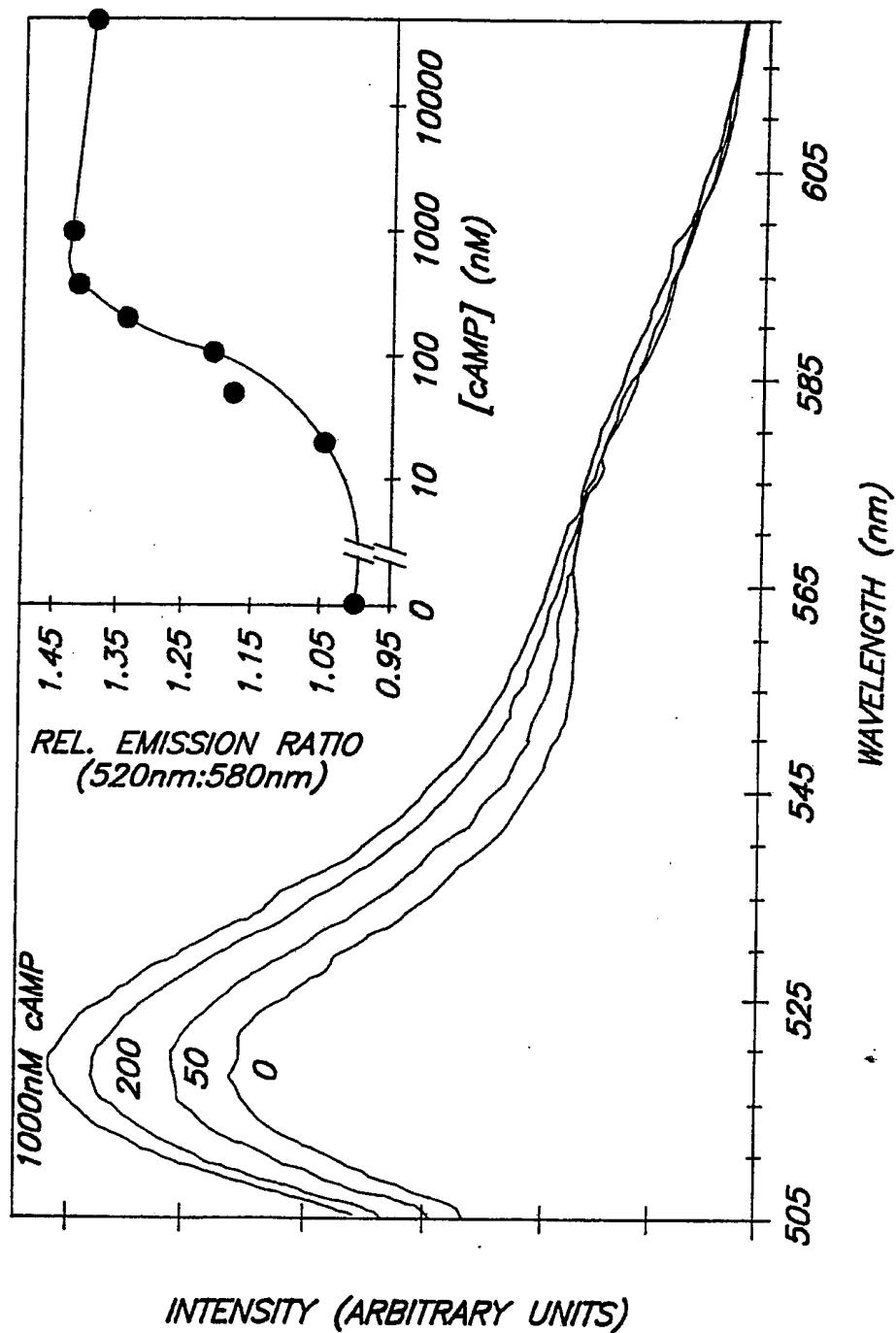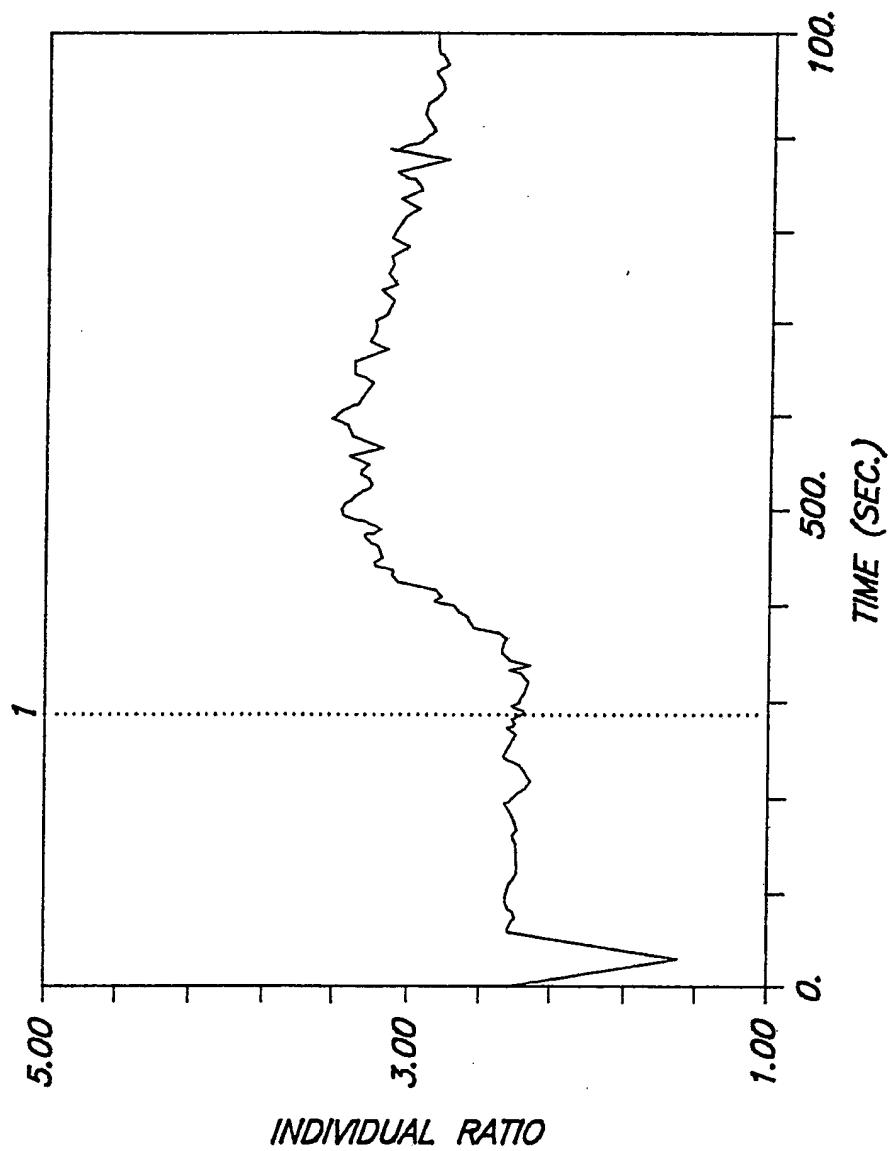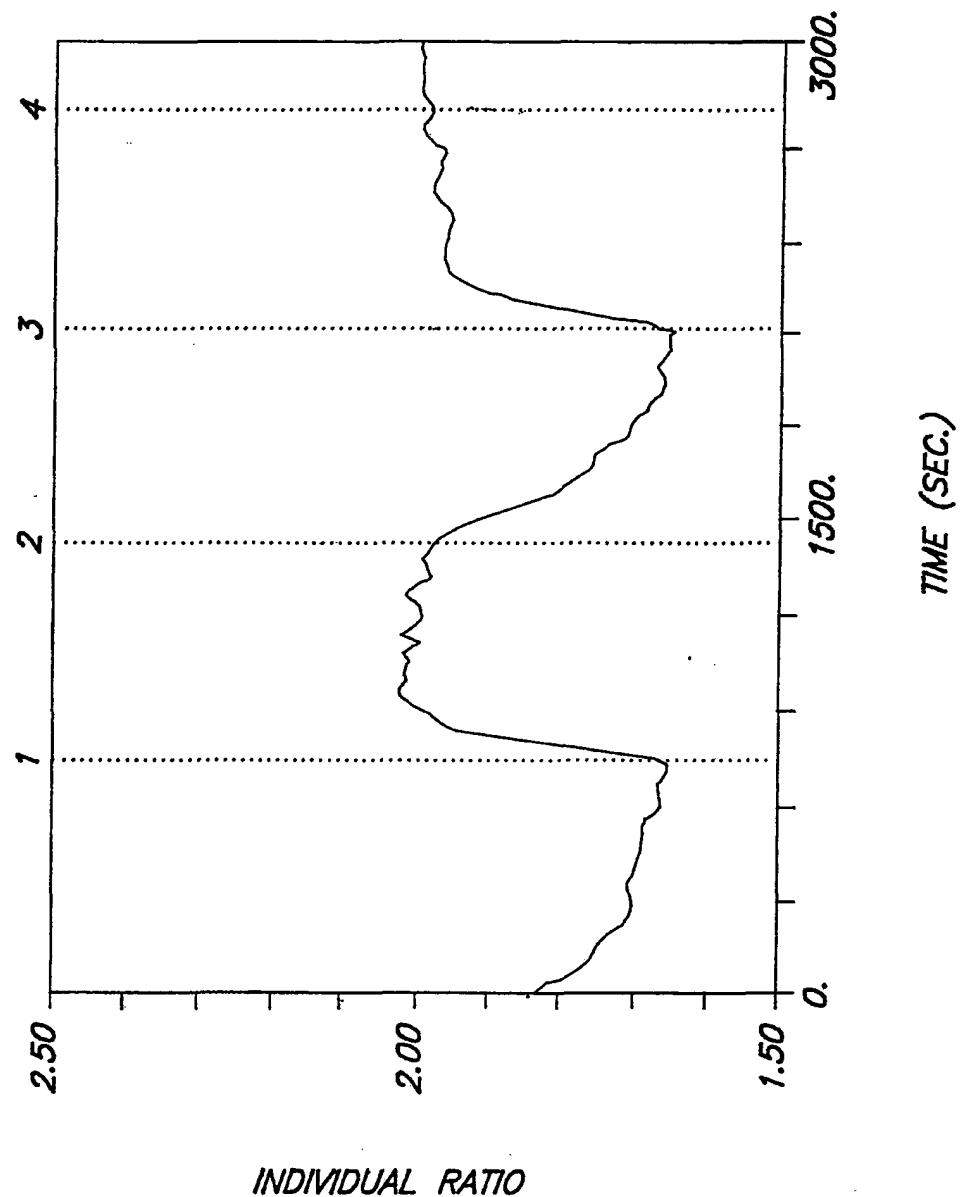
FIG. 2

FIG. 3

FIG. 4

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) •

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. CL.: 435/7.21; 436/501, 546

IPC(5): C12Q 1/68; G01N 33/566, 33/533

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S. CL.	435/7.21; 436/501, 436/546

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

APS, MEDLINE SEARCH TERMS: energy transfer, CAMP dependent protein kinase, association, dissociation, subunit

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category •	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X Y	US,A, 4,868,103, (STAVRIANOPOULOS ET AL.) 19 September 1989, see Col.5, lines 20-60.	1,2,5-7,14-16,19 22-24 & 31
X Y	US,A, 4,828,981, (MAGGIO) 09 May 1989, see entire document.	1-45 1,5-7,14-16,22-24 & 31
Y	J. BIOLOGICAL CHEMISTRY, Vol. 261, issued 26 April 1986, Fletcher et al., "Cytochemical identification of the regulatory subunit of the cAMP-dependent protein kinase by use of fluorescently labeled catalytic subunit," pages 5504-5513, see entire document.	1-45 17,18
Y	J. BIOLOGICAL CHEMISTRY, Vol. 264, issued 05 August 1989, Woodford et al., "Expression and characterization of mutant forms of the type I regulatory subunit of cAMP-dependent protein kinase," pages 13321-13328, see entire document.	11-13,28-30,35-37& 43-45

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 SEPTEMBER 1991	29 NOV 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	M.P. WOODWARD

III. DOCUMENTS CONSIDERED T BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	J. BIOLOGICAL CHEMISTRY, Vol. 261, issued 25 February 1986, McEvily et al., "Subunit equilibria of porcine heart citrate synthase," pages 2593-2598, see entire document.	1,2,5-7,14-16,19, 22-24 & 31 / 1-7,12-16,19,22-24 30,31,33,37,38,41& 45
Y	BIOCHEMISTRY, Vol. 28, issued 1989, First et al., "Fluorescence energy transfer between cysteine 199 and cysteine 343: evidence for MgATP-dependent conformational change in the catalytic subunit of cAMP-dependent protein kinase," pages 3606-3613, see entire document.	1-45
Y	BIOCHEMISTRY, Vol. 28, issued 1989, First et al., Selective modification of the catalytic subunit of cAMP-dependent protein kinase with sulfhydryl-specific fluorescent probes," pages 3598-3605, see entire document.	1-45
Y	EUR. J. BIOCHEM., Vol. 188, issued 1990, Buchler et al., "Regulation of gene expression by transfected subunits of cAMP-dependent protein kinase," pages 253-259, see entire document.	11-13,17,18,28-30 35-37&43-45